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Attorney Docket: 104/50108
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: ANTONIO NANCI ET AL.

Serial No.: 09/868,310 Group Art Unit: 1636

Filed: OCTOBER 1, 2001 Examiner: Celine X. QIAN

Title: IN VIVO MODEL USING CONTINUOUSLY ERUPTING SYSTEM

RESPONSE TO RESTRICTION REQUIREMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In response to the Requirement for Restriction mailed February 11, 2003, Applicants provisionally elect, with traverse, to prosecute the claims of Group I, namely Claims 1 to 3 drawn to an *in vivo* rodent model comprising a mandibular incisor and a window in an alveolar bone.

Reconsideration of the Restriction Requirement is however respectfully requested. The Examiner maintains in her Requirement for Restriction that the "special technical feature" relating the claims of Groups I-VIII, namely the *in vivo* rodent model comprising a mandibular incisor and a window in an alveolar bone, is anticipated and lack inventive step over McKee and Warshawsky.

The Applicant respectfully submits that the *in vivo* rodent model as claimed in claim 1 differs from the *in vivo* rat model described by McKee and Warshawsky. A first difference is the location of the window. In McKee and Warshawsky, the window is located in the labial alveolar bone Further differences are detailed in an article entitled "In vivo Model for the

Experimental Manipulation of Calcified Tissues: A Surgical Approach for Accessing the Odontogenic Organ and Associated Tissues of the Rat Incisor" authored by the inventors of the present invention and published in the Journal of Histochemistry & Cytochemistry. A copy of this Article is enclosed herewith as Exhibit 1. This article teaches that:

> "These approaches [McKee and Warshawsky...] were designed to target both secretory and maturation stage ameloblasts as well as the adjacent enamel. However, because of the confined and narrow space separating the alveolar bone and the underlying enamel organ, previous surgical procedures caused considerable damage to tooth structure and required waiting for undamaged tissues to move forward under the window before further experimentation could be accomplished. Moreover, they did not allow targeting of the precursor cells located at the apical end of the tooth, where inductive molecular events take place, and were therefore restricted to specific developmental stages of the enamel organ. Most importantly, because enamel forms in a secluded environment regulated and maintained, at least in part, by ameloblasts, any interruption in the integrity of the enamel organ inevitable leads to an alteration in the physiological parameters of enamel." (Emphasis added)

In sharp contrast, the presence of the window on the buccal side of the alveolar bone, overlying the incisor's apex or along radicular surface of the incisor as in claim 1 of the present invention, causes damage to the odontogenic organ in only a very few cases for which damages were, in general, due to anatomic variability.

For the reasons mentioned above, the model as claimed in claim 1 differs from the model described by McKee and Warshawsky and that therefore, the claims of Group 1 are not anticipated and have inventive step over McKee and Warshawsky. Moreover, this absence of anticipation provides a special technical feature which is common to the claims of

Groups I to VIII forming therefore a single general inventive concept as required by PCT Rule 13.1.

Therefore, it is respectfully submitted that these eight groups (Group I to VIII) are closely connected together by a common special technical feature and that the search and examination of claims 1 to 14 of the application can be made without serious burden on the Examiner.

From the foregoing, the eight (8) groups of invention should therefore be rejoined and examination is solicited on claims 1 to 14 as pending.

In the event that there are any questions concerning this response, or the application in general, the Examiner is respectfully urged to telephone the undersigned so that prosecution of the application may be expedited.

If necessary to effect a timely response, this paper should be considered as a petition for an Extension of Time sufficient to effect a timely response, and please charge any deficiency in fees or credit any overpayments to Deposit Account No. 05-1323 (Docket #104/50108).

Respectfully submitted,

June 11, 2003

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Exhibit 1

Volume 47(3): 323–336, 1999
The Journal of Histochemistry & Cytochemistry
http://www.jhc.org

ARTICLE

In Vivo Model for the Experimental Manipulation of Calcified Tissues: A Surgical Approach for Accessing the Odontogenic Organ and Associated Tissues of the Rat Incisor

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SUMMARY The tooth organ is extensively used in developmental biology to investigate organogenesis and cell differentiation. It also represents an advantageous system for the study of the various cellular and extracellular matrix events that regulate the formation of both collagenous and noncollagenous calcified tissues. This article describes an in vivo surgical approach to access and experimentally manipulate the tooth organ and supporting tissues of the rat incisor. By use of a dental drill, a "window" was created through the alveolar bone on the buccal aspect of the hemimandible at the apical end of the incisor. It is at this site that epithelial and mesenchymal precursors are situated and undergo cellular differentiation to give rise to cells of the odontogenic organ. Active bone remodeling is also observed in this area to accommodate posterior growth of the tooth. An osmotic minipump connected to the bony window through an outlet catheter was used for controlled and continuous administration of experimental agents over a predetermined period of time. To validate the model, vinblastine sulfate, fetuin–gold, and dinitrophenylated albumin were thus infused. The animals were then sacrificed and the hemimandibles were processed for histological and immunocytochemical analyses. The effects of the drug and the presence of tracers were restricted to the treated hemimandible and were found in the enamel organ and pulp, as well as in the tooth supporting tissues. Cellular changes typically associated with the administration of vinblastine were obtained, and tracers were localized both in the extracellular milieu and within the endosomal/lysosomal elements of cells. These results suggest that this new surgical approach could serve as an advantageous in vivo model in which various chemical agents, therapeutic drugs, molecular probes are locally administered to study the molecular events that regulate calcified tissue formation.
(J Histochem Cytochem 47:323–336, 1999)

KEY WORDS
osmotic minipump
experimental manipulation
tracers
vinblastine sulfate
calcified tissues
odontogenic organ
incisor
rat

TOOTH DEVELOPMENT is mediated by reciprocal inductive interactions between neural crest-derived ectomesenchyme cells and the oral epithelium (reviewed in Slavkin 1990; Ruch et al. 1995; Thesleff et al. 1996). Cells of epithelial origin differentiate into ameloblasts which synthesize and secrete the noncollagenous matrix of enamel, and ectomesenchymal cells give rise to odontoblasts, which produce the collagen-containing dentin matrix. Epithelial and/or ectomesenchymal cells may also differentiate into cementoblasts, which deposit cementum, a tissue that comprises both noncollagenous and collagenous proteins (reviewed in Bosshardt and Nanci 1998). Furthermore, the eruption and growth of the tooth involve the participation of surrounding/supporting bone and periodontal tissues. More specifically, alveolar bone remodeling occurs around the apical portion of the rat incisor, allowing posterior growth of the tooth (Marks and Schroeder 1996). Formation and mineralization of all the above calcified tissues results from a tightly regulated series of cellular events and interactions between the organic and inorganic phases of the extracellular matrix (reviewed in

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Received for publication August 19, 1998; accepted October 13, 1998 (8A4758).

0022-1554/99/$3.30

Nanci and Smith 1992; Freemont 1993; Linde and Goldberg 1993; Goldberg et al. 1995; Smith and Nanci 1995; Zeichner-David et al. 1995; Gehron Robey 1996; McKee and Nanci 1996b; Bosshardt et al. 1998; Butler 1998; Robinson et al. 1998; Triffitt et al. 1998).

The continuously erupting rat incisor has been extensively used to study the cellular and extracellular matrix events involved in odontogenesis because all stages of development can be found in a single tooth and it exhibits many similarities to human tooth formation (Leblond and Warshawsky 1979; Warshawsky et al. 1981). Facing epithelial and mesenchymal cells undergo a series of differentiation and phenotypic changes which have been precisely mapped along the linear axis of the tooth (Smith and Nanci 1989). Anatomically, much of the rat incisor lies in the body of the mandible. In addition, its odontogenic organ, responsible for renewal of all epithelial cells of the tooth organ (Smith and Warshawsky 1975a), is situated in a very active but secluded area, making the incisor an excellent model for local and selective targeting of dental tissue cells.

Despite the advantages offered by the rat incisor, there have been only limited attempts to develop experimental approaches for direct manipulation of the cellular and matrix events in this tooth. Some experiments have involved removal of blocks of bone and apical portions of the incisor to study cell activity during bone remodeling and tooth eruption (Redondo et al. 1995; Berkovitz and Thomas 1969; Berkovitz 1971a,b). Others have used the surgical creation of a hole through the alveolar bone at the labial surface of the tooth to obtain direct access to the underlying tissues (McKee and Warshawsky 1984; Eisenmann et al. 1989; McKee 1993; McKee and Nanci 1996a). These approaches were designed to target both secretory and maturation stage ameloblasts as well as the adjacent enamel. However, because of the confined and narrow space separating the alveolar bone and the underlying enamel organ, previous surgical procedures caused considerable damage to tooth structure and required waiting for undamaged tissues to move forward under the window before further experimentation could be accomplished. Moreover, they did not allow targeting

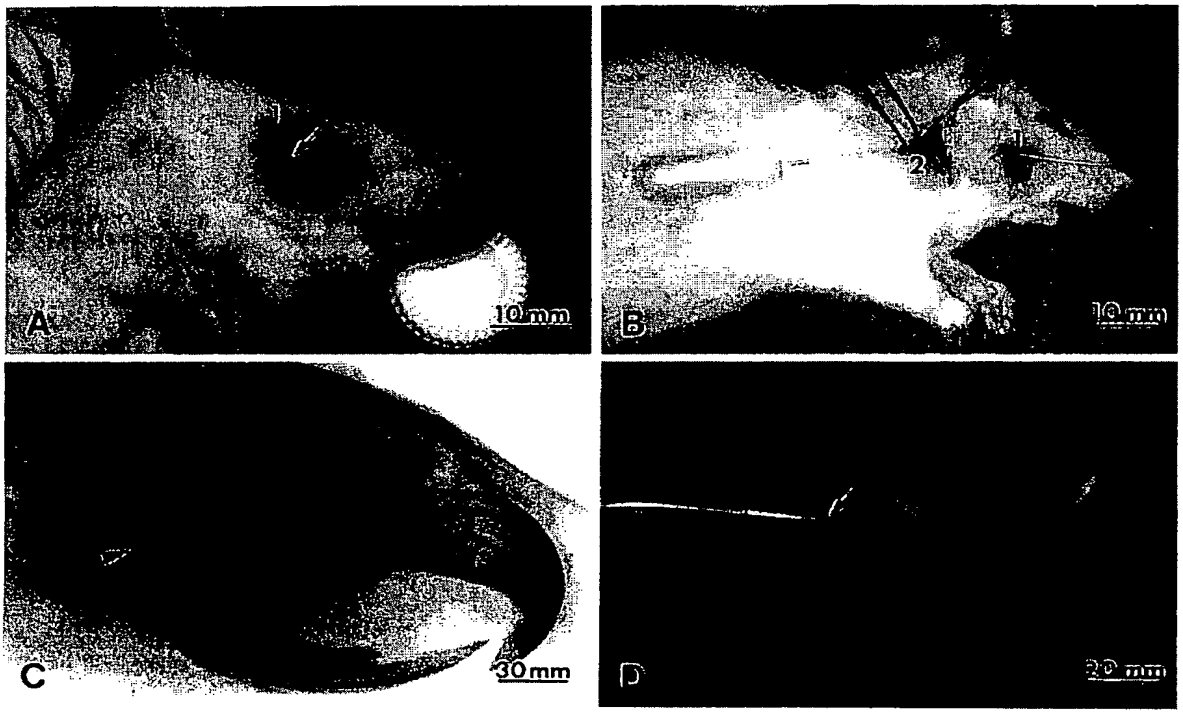

Figure 1 Photographs illustrating (A) the first incision (1) made through the skin and muscle for drilling a surgical window (arrow) through the alveolar bone overlying the connective tissue surrounding the apex of the rat incisor, and (B) the second skin incision (2) for the dorsal positioning of the osmotic minipump. The pump cathether passes below the masseter. (C) Microradiograph showing the position of the metal tip of the catheter with respect to the apical portion (arrowhead) of the incisor. (D) Photograph of a hemimandible after perfusion, with the metal tip of the catheter fitted into the bony window (arrowhead).

of the precursor cells located at the apical end of the tooth, where inductive molecular events take place, and were therefore restricted to specific developmental stages of the enamel organ. Most importantly, because enamel forms in a secluded environment regulated and maintained, at least in part, by ameloblasts, any interruption in the integrity of the enamel organ inevitably leads to an alteration in the physiological parameters of enamel.

In this study we investigated whether the rat mandibular incisor could be exploited as an experimental model for local and selective targeting of the odontogenic organ and its associated periodontal tissues. A surgical technique was developed to create a "window" in the alveolar bone overlying the apex of the rat incisor, and an osmotic minipump was utilized to deliver specific experimental agents. Vinblastine sulfate and two tracer molecules, fetuin–gold and albumin tagged with dinitrophenol, were utilized to validate the efficiency of the surgical approach in targeting cells of the tooth organ. Minipumps have previously been used in dogs to deliver bafilomycin A_1, an inhibitor of vacuolar H^+-ATPases in osteoclasts, to block alveolar bone resorption and tooth eruption (Sundquist and Marks 1994; Marks and Sundquist 1995). They are advantageous compared to microinjection because they can deliver, in a controlled and continuous manner, relatively large amounts of experimental agents through a bony window. This in vivo experimental model may prove advantageous for future experiments in which various drugs and molecular probes are applied to elucidate the cellular and biochemical events that regulate calcified tissue formation.

Materials and Methods

Surgical Procedure

Male Wistar rats weighing 100 ± 10 g (Charles River Canada; St-Constant, QC, Canada) were anesthetized with an IP injection of 0.06 ml Somnotol (sodium pentobarbital; MTC Pharmaceuticals, Cambridge, ON, Canada). The vestibular

Figure 2 Scanning electron microscopic image of the hole drilled through the mandibular bone. The hemimandible was fixed, digested with sodium hypochlorite, and examined in the humid state with a variable pressure instrument. The buccal (B) wall of the hemimandible is partially removed, revealing the opposing lingual (L) aspect. Material infused into the hole diffuses along the periodontal space towards (arrows) the apical end of the incisor.

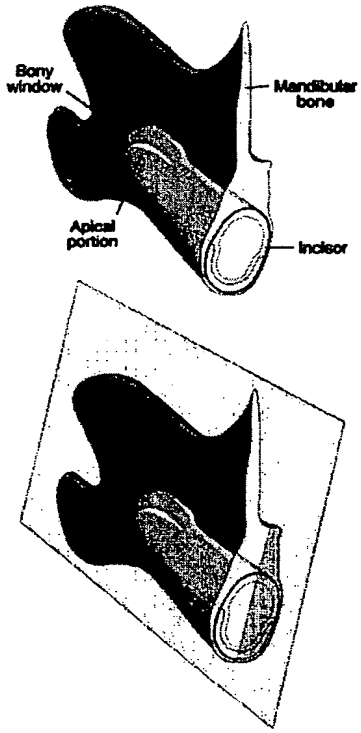

Figure 3 Schematic illustrations of the posterior aspect of the hemimandible showing the relationship of the apical end of the incisor to the bony window (top) and the plane of section, passing along the longitudinal axis of the tooth and across the window (bottom), used to obtain Figures 4 and 5.

surface of the right mandibular ramus was surgically exposed as follows (Figure 1). An incision about 8 mm long was made through the skin with fine scissors to access the muscle layer underneath, according to an imaginary line joining the auditory meatus and the lip commissure. The fibers of the masseter were separated along their longitudinal axis with a scalpel blade. A periosteal separator was then used to elevate the periosteum and expose the underlying bony surface of the ramus. The musculature was retracted with a plastic ring made from an embedding BEEM capsule size 1 (Marivac; Halifax, NS, Canada). The surgical area was kept moist with rinses of physiological saline. A slow-speed dental drill equipped with a carbide round burr size 6 was used to create a hole through the alveolar bone (Figure 1A). The bony window was placed approximately 2 mm anterior to the posterior border of the ramus and slightly superior to the bony elevation at the apical end of the incisor. Penetration through the alveolar bone into the periodontal space around the apex was established by slight bleeding on breakthrough. The burr was then removed and a cotton swab was placed over the hole until the bleeding stopped. A third incision through the skin in the neck area was made to accommodate an Alzet 1003D or 2001D osmotic minipump (Alza Corporation; Palo Alto, CA) (Figure 1B). The 1003D model has a capacity of 90 µl and a flow rate of 1 µl/hr for 3 days, and the 2001D has a capacity of 234 µl and a flow rate of 8 µl/hr for 24 hr. The pump was tunneled into a subcutaneous pouch on the back of the animal and connected to the bony hole using a vinyl tubing and a metal catheter made from a 20G1 syringe needle (Becton–Dickinson; Rutherford, NJ). The tubing was passed underneath the masseter muscle and through the neck area. His-

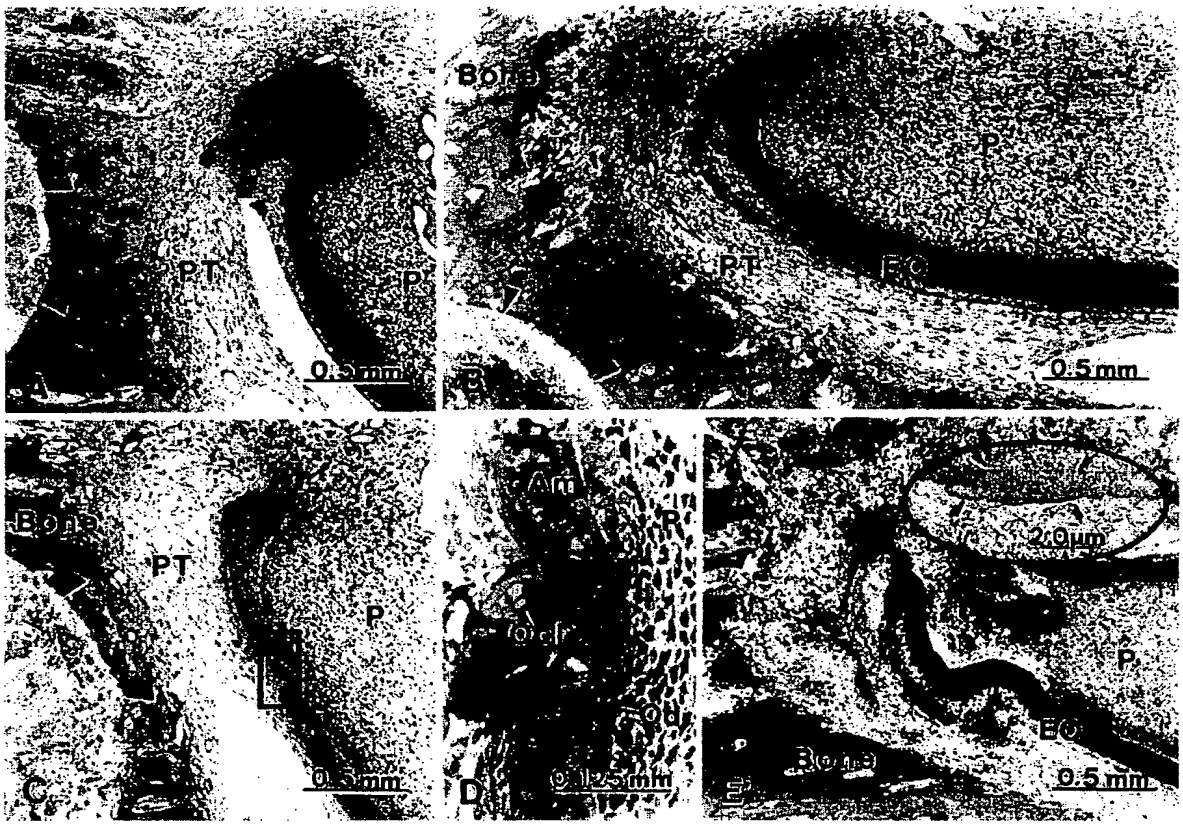

Figure 4 Light micrographs illustrating the relation of the bony window to the enamel organ (EO) of the incisor. Sections were cut along the longitudinal axis of the tooth (see Figure 3). (A,B) In the majority of cases, the hole (arrowhead) is positioned a short distance away from the enamel organ, resulting in no mechanical damage to the tooth. (C,D) Occasionally, bone debris (asterisk), created during drilling, compresses part of the tooth surface, causing minor focal tissue changes. (D) Enlargement of the boxed area in C. (E) If the mandibular reference points are not respected, drilling can occur over the tooth organ inducing major tissue damage. However, alterations are generally localized to the area where the burr contacts the tooth. (Inset) Occasionally, a layer of matrix resembling acellular afibrillar cementum (between small arrows) is deposited at the ameloblast surface near the site of damage. Am, ameloblasts; Ocl, osteoclast; Od, odontoblasts; P, pulp; PT, periodontal tissue.

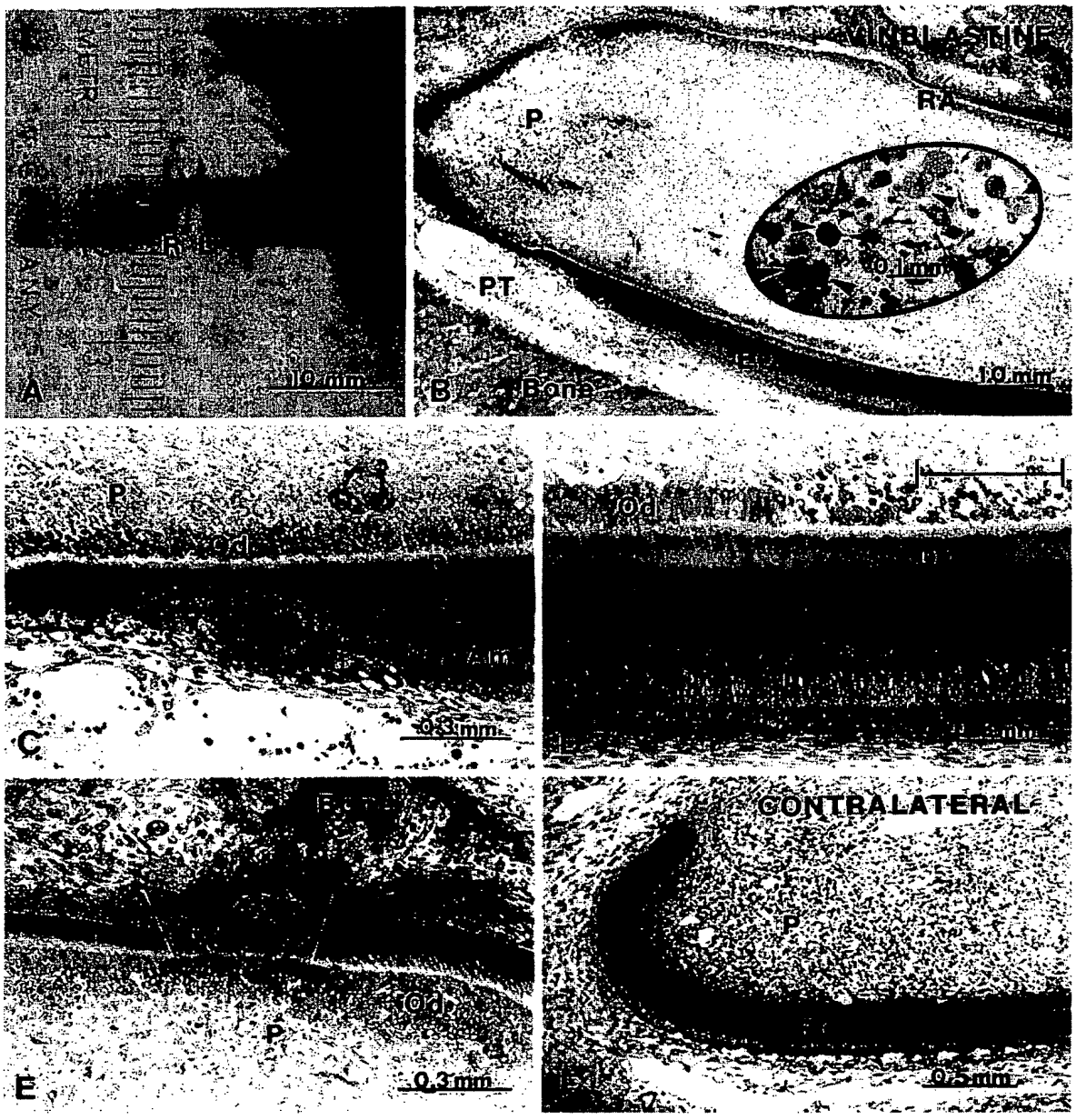

Figure 5 Micrographs illustrating the alterations induced by infusion of vinblastine sulfate for 3 days. (A) The incisor on the treated side (R) is shorter than the contralateral (L) tooth. (B) The drug has diffused throughout the tooth, affecting the enamel organ (EO), the pulp (P), and the root analogue surface (RA). (Inset) Many mitotic figures (arrowheads) are present in the pulp. (C) The organization of the enamel organ and the production of enamel matrix (E) by ameloblasts (Am) are affected in the early part of amelogenesis (compare C and D). (D) Ameloblasts that differentiated before administration of the drug show a normal organization. However, groups of odontoblasts (Od) appear to have degenerated (bracket). (E) A focal alteration of dentin (D) production and mineralization (dashed lines) is sometimes observed on the root analogue surface. (F) No structural changes were observed on the contralateral tooth. PT, periodontal tissue.

toacryl glue (B. Braun Melsungen AG, Germany/Sherwood DG, Dorval, QC, Canada) and bone cement (Zimmer; Warsaw, IN) were used to immobilize the metal catheter against the bone surface and maintain its tip in the bony hole. The animals were then sutured and the surgical site was cleaned and disinfected with 70% ethanol. After surgery, X-ray photographs of the rat mandibles were taken to verify that the catheter was well in place (Figure 1C) and the rats were allowed to recover under observation. Some of the animals received 0.01 ml of buprenorphine HCl 0.3 mg/ml (Temgesic; Reckitt & Colman, Hull, UK) as analgesic immediately after surgery. All animal procedures and experimental protocols described above were in strict accordance with guidelines of the Comité de Déontologie de l'Expérimentation sur les Animaux of Université de Montréal.

Administration of Vinblastine Sulfate

Six rats were implanted with Alzet osmotic minipumps model 1003D filled with a solution of 0.17 mg/ml of vinblastine sulfate in physiological saline (Sigma Chemical; St Louis, MO). The minipumps were connected to the vinyl tubing, also filled with the drug, and incubated in sterile saline at 37C for 3 hr before placement as described above.

Administration of Tracers

Groups of two rats were implanted with saline-preincubated Alzet 2001D minipumps filled with fetuin-gold or DNP-tagged albumin for 24-hr infusion. The fetuin-gold complex (particles of ~15 nm in diameter; 25 μg/ml) was purchased from EY Laboratories (San Mateo, CA). Bovine serum albumin (Sigma) was tagged with dinitrophenol (DNP) using the method of Little and Eisen (1967), as reported previously (Ghitescu and Bendayan 1993; Nanci et al. 1996a).

Tissue Processing

On the third day of infusion of vinblastine sulfate and at 24 hr for the tracers, the animals were anesthetized with an IP injection of 0.25 ml of 20% chloral hydrate (Sigma) and sacrificed by intravascular perfusion through the left ventricle. The vasculature was prerinsed with lactated Ringer's solution (Abbott Laboratories; Montreal, QC, Canada) for about 30 sec (until the liver blanched), followed by perfusion with a fixative solution consisting of 1% glutaraldehyde in 0.08 M sodium cacodylate buffer containing 0.05% $CaCl_2$, pH 7.3, for 20 min. Both hemimandibles were dissected out and immersed in the fixative overnight at 4C. They were then

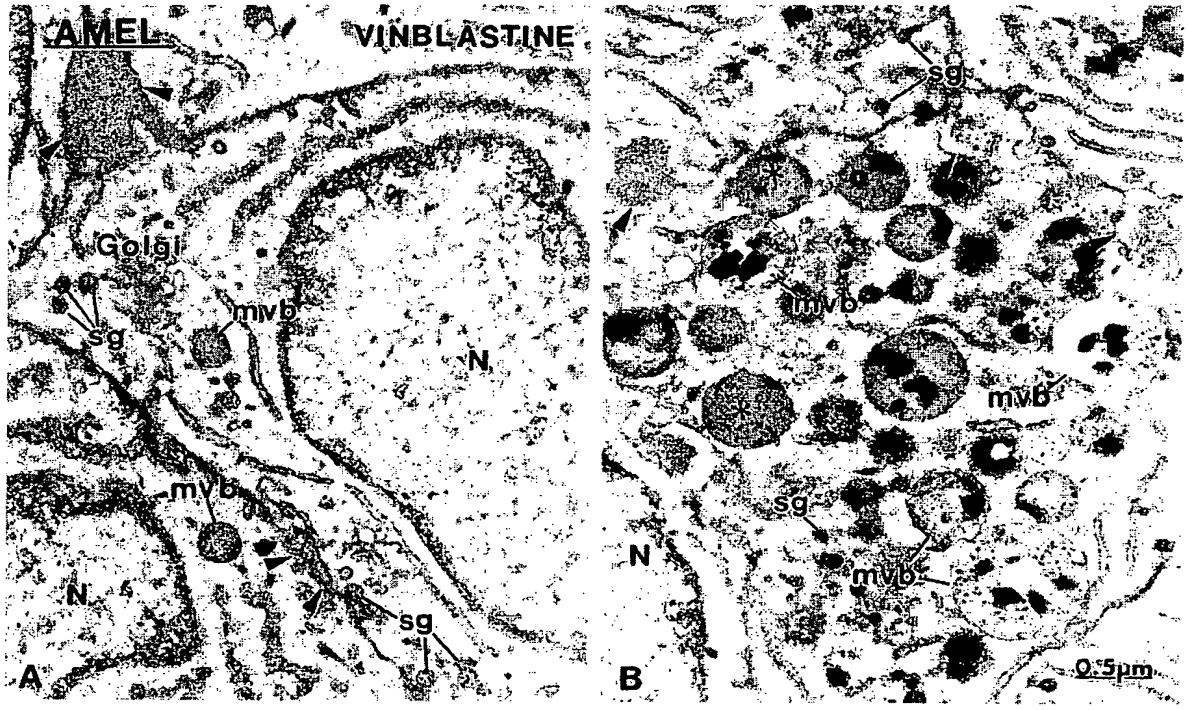

Figure 6 Immunocytochemical preparations with anti-amelogenin (AMEL) antibody. (A,B) Local infusion of vinblastine sulfate through the bony window results in the typical cellular changes induced by systemic injection of this anti-microtubule agent (see Nanci et al. 1987). The Golgi apparatus (Golgi) is fragmented, secretory granules (sg) accumulate throughout the cell body, and many lysosome-like elements (asterisks) and multivesicular bodies (mvb) appear. Immunoreactivity for amelogenin is present in all these compartments. Secretory stage ameloblasts also release enamel proteins (arrowheads) ectopically along their basolateral surfaces. N, nucleus.

washed in 0.1 M sodium cacodylate buffer containing 0.05% $CaCl_2$, pH 7.3, and decalcified in 4.13% EDTA for 14 days at 4C (solution was changed every 2 days) (Warshawsky and Moore 1967). The hemimandibles were subdivided into segments and washed again in 0.1 M sodium cacodylate buffer. Half of the segments were postfixed with 2% potassium ferrocyanide-reduced osmium tetroxide for 2 hr at 4C (Neiss 1984). All segments (osmicated and nonosmicated) were then dehydrated in a graded alcohol series and embedded in LR White resin (Marivac) or dehydrated in a graded acetone series and embedded in Taab epoxy resin (Marivac). Liver, duodenum, kidney, and parotid gland were also harvested and processed for embedding in LR White or epoxy resin.

Each tooth segment was oriented for sectioning along its longitudinal axis (see schematic illustration in Fig. 3). One-μm-thick sections were cut with glass knives on a Reichert–Jung Ultracut E ultramicrotome and stained with toluidine blue. Thin sections were cut with a diamond knife and mounted on 200-mesh nickel grids having a carbon-coated Formvar film. Selected sections were processed for postembedding colloidal gold immunocytochemistry (reviewed in Bendayan 1995) for detection of enamel proteins (amelogenin; Nanci et al. 1996b) or DNP-tagged albumin, whereas fetuin–gold was directly observed. All grids were stained with uranyl acetate and lead citrate for examination in a JEOL JEM-1200EX-II transmission electron microscope operated at 60 kV.

Immunolocalization of Dinitrophenol-tagged Albumin and Amelogenin

Sections from osmicated samples were first treated with sodium metaperiodate (Bendayan and Zollinger 1983) for 30 min (LR White) or 1 hour (epoxy resin) and rinsed with distilled water. All sections were floated for 15 min onto a drop of 0.01 M PBS containing 1% ovalbumin (Oval). They were transferred onto a drop of rabbit anti-DNP antibody diluted 1:150 (Dako; Carpinteria, CA) or recombinant M179 mouse amelogenin antibody diluted 1:300 for 1 hr, washed with PBS, and refloated on PBS–Oval for 15 min. Antibody binding sites were then revealed by incubating the tissue sections with protein A–gold complex for 30 min. The protein A–gold was prepared as described by Bendayan (1995) using colloidal gold particles of 8 or 14 nm (Frens 1973). For detection of enamel proteins, a chicken egg yolk antibody to rat 24-kD amelogenin was also used, as previously described (Nanci et al. 1996b). Briefly, the sections were floated on a drop of a blocking solution [0.5% BSA (Sigma), 0.1% gelatin (Merck; Darmstadt, Germany), 0.005% Tween-20 (Bio-Rad; Richmond, CA) and 0.5 M NaCl (BDH; Poole, UK)

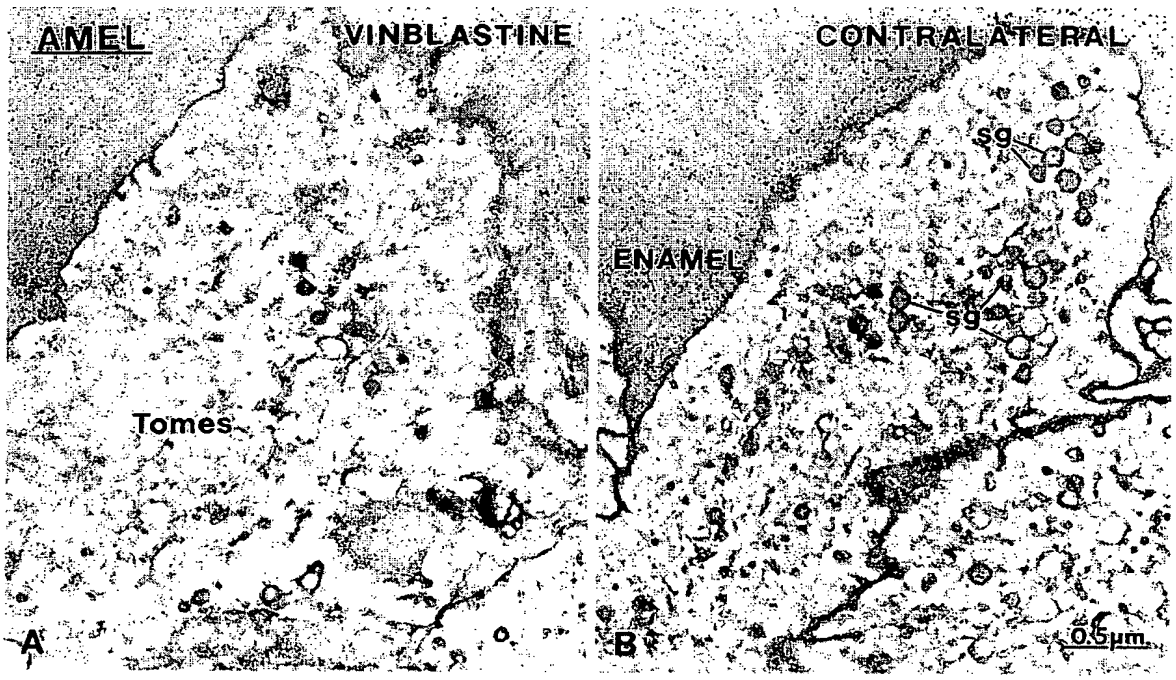

Figure 7 Comparative immunocytochemical preparations with anti-amelogenin (AMEL) antibody, showing the apical portion of secretory stage ameloblasts from (A) the vinblastine-treated and (B) the untreated contralateral incisor. As expected, vinblastine results in the loss of secretory granules (sg) from Tome's processes (Tomes) only on the treated side, demonstrating the localized action obtained by infusing drugs through the bony window. Secretory granules and the enamel matrix are immunoreactive for amelogenin.

dissolved in 0.01 M PBS, pH 7.4] for 15 min. They were then incubated on a drop of a primary egg yolk antibody (diluted 1:150 in PBS 0.01 M) for 3 hr, washed with PBS, and refloated on the blocking solution for another 15 min. Afterward, they were incubated with a rabbit anti-chicken IgG antibody (diluted 1:2000) (Cappel Research Products; Scarborough, ON, Canada) for 1 hr. They were washed in PBS, refloated on the blocking solution for 15 min, and incubated with protein A–gold complex for 30 min. For controls, grids were incubated with secondary antibody followed by protein A–gold, or with protein A–gold alone. All incubation steps were carried out at room temperature. The grids were finally rinsed with PBS, distilled water, and then air-dried.

Scanning Electron Microscopy

Two animals were sacrificed by anesthetic overdose after creation of the bony window. The hemimandibles were dissected and cleaned of soft tissue, fixed overnight by immersion with 1% glutaraldehyde in 0.08 M cacodylate buffer containing 0.05% $CaCl_2$, pH 7.3, and washed in 0.1 M sodium cacodylate buffer. Residual soft tissue was removed by digestion with 6% sodium hypochlorite. They were then washed and kept in distilled water until observation in the humid state with an Hitachi S-3500N variable-pressure scanning electron microscope operated in the backscattered mode at 20 kV and 40 Pa pressure.

Results

Surgical Procedure

Initial studies aimed at establishing the appropriate position of the bony window identified the posterior border of the ramus and the bony elevation overlying the apical end of the incisor as reliable reference points for drilling. Passing the minipump catheter below the masseter muscle and immobilization of its metal tip with Histoacryl glue and bone cement resulted in firm anchorage. The positioning and stability of the catheters in the bony window were confirmed on X-rays (Figure 1C). Visual inspection at dissection time was also used to confirm that the catheter was still in position (Figure 1D) and to rule out any block-

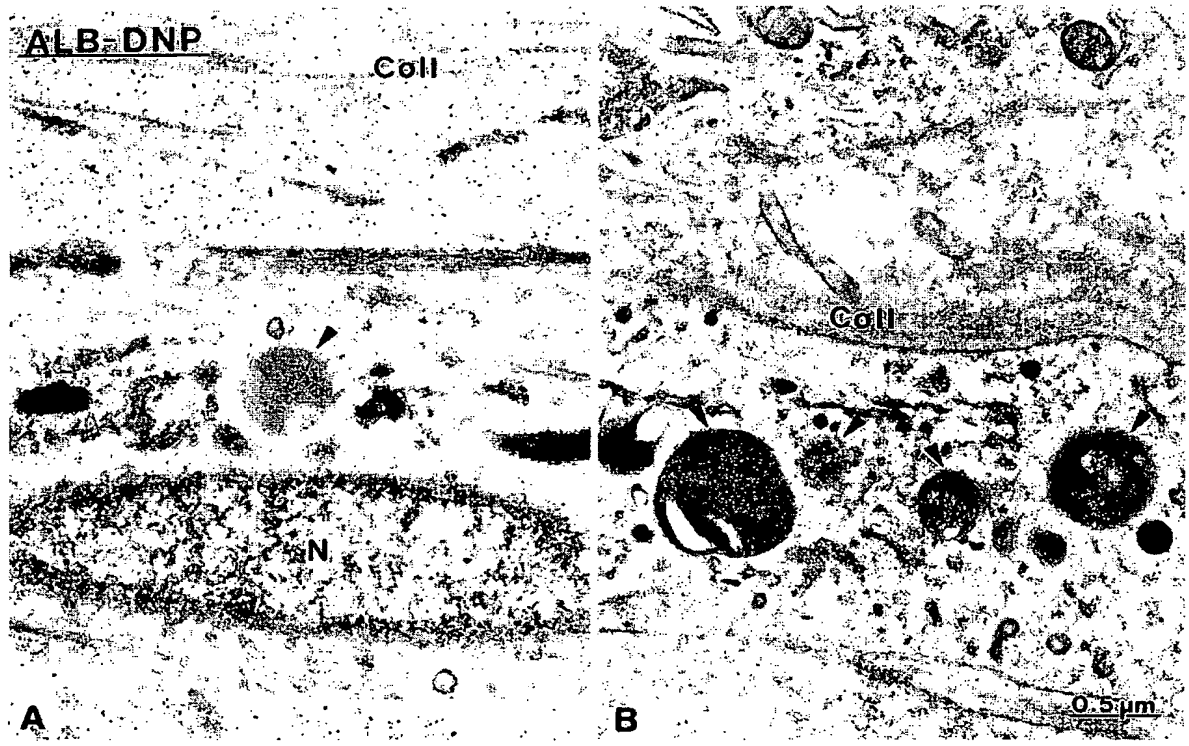

Figure 8 Immunocytochemical preparations illustrating the distribution of infused albumin–dinitrophenol (ALB–DNP) in the connective tissue surrounding the enamel organ. The tracer is taken up by fibroblasts into their lysosomal/endosomal elements (arrowheads). More tracer molecules are immunodetected extracellularly (A) near the bone window than (B) at a distance (~3 mm) from the hole. Coll, collagen fibrils; N, nucleus.

age by tissue debris and blood clotting. The reference points allowed proper positioning of the bony window and no damage to the enamel organ (Figures 2, 4A, and B) in eight of 10 animals used to validate the surgical procedure before the present drug and tracer study. Inaccurate anterior positioning resulted either in compression by bone debris (Figures 4C and 4D) or focal destruction (Figure 4E) of the enamel organ, while complete perforation of the thin alveolar bony walls occurred posteriorly. Inflammatory cells infiltrated the damaged enamel organ and a cementum-like substance was deposited in the forming extracellular matrix (Figure 4E, inset) (Bosshardt and Nanci 1998). Surprisingly, the enamel organ surrounding the site of tissue damage appeared to develop normally.

Morphological Alterations Induced by Vinblastine Sulfate

Continuous exposure of the dental organ to vinblastine sulfate for 3 days affected tooth eruption such that the treated incisor was about 1 mm shorter than the contralateral tooth (Figure 5A). It is noteworthy that none of the rats used in initial studies aimed at validating the surgical procedure (no minipumps placed), as well as those used for tracer studies, showed a difference in length between the treated and the contralateral untreated incisor. Cell organization and function were also altered (Figures 5B–5E). The Golgi apparatus of secretory stage ameloblasts was fragmented, clusters of secretory granules were found throughout the cell body (Figure 6A), and endosomal/lysosomal elements were abundant (Figure 6B). These cell compartments were all immunoreactive for amelogenin (Figure 6). Tomes' processes showed very few or no secretion granules (Figure 7). These were abundant and intensely immunoreactive for amelogenin in contralateral incisor ameloblasts (Figure 7B). The organization and shape of early secretory stage ameloblasts were disrupted and there was ectopic release of enamel proteins along their basolateral surfaces (Figure 6A). Groups of odontoblasts showed signs of degeneration, even though the

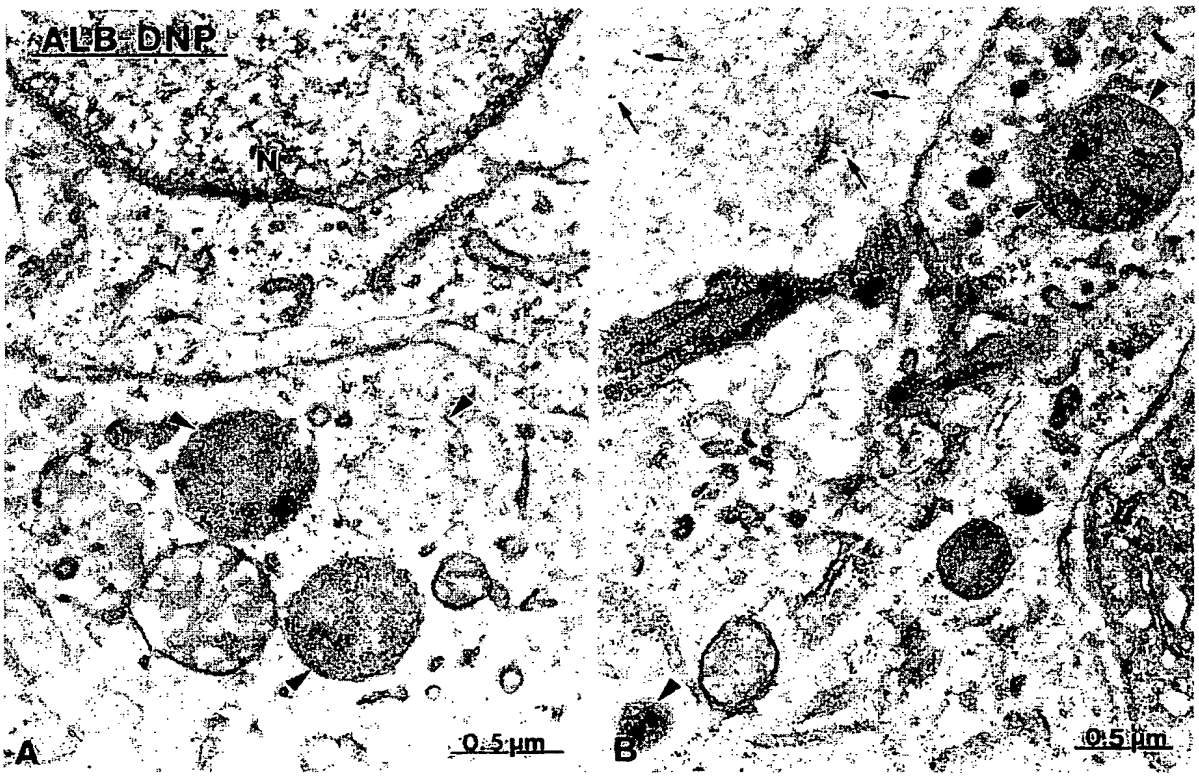

Figure 9 Some of the infused albumin–dinitrophenol (ALB–DNP) tracer diffuses through the apical foramen of the incisor into the pulp chamber, where it is taken up into endosomal/lysosomal elements (arrowheads) of (A) odontoblasts and (B) resident mesenchymal cells. Some tracer is also immunodetected extracellularly (arrows) among the pulp cells. N, nucleus.

appearance of the overlying dentin was normal (Figure 5D). There were abundant mitotic cells in the pulp and periodontal tissue (Figure 5, inset). In one case, focal alteration of dentin production and mineralization on the root analogue surface were also observed (Figure 5E). Tissues of the contralateral hemimandible appeared normal and showed none of the above cell and matrix alterations (Figures 5F and 7B). The duodenum, kidney, and parotid cells also revealed no structural changes (data not illustrated).

Distribution of Tracers

Dinitrophenol-tagged albumin was found in endosomal/lysosomal elements of periodontal fibroblasts and in the interstitial fluid surrounding them (Figure 8), and less frequently in pulp cells and odontoblasts (Figure 9). There was also abundant immunoreactivity around osteoblasts and in osteoid near the site of infusion (Figure 10), but the density of labeling associated with bone diminished significantly away from the bony window. DNP-albumin was also detected in multivesicular bodies of early secretory stage ameloblasts (Figure 11). Although some sporadic labeling was found in liver cells, only a few randomly distributed gold particles were observed on tissue sections of the contralateral tooth incubated with anti-DNP antibody. Control incubations with protein A-gold alone resulted in a few gold particles randomly distributed throughout the tissue section.

Fetuin-gold was predominantly found at the site of drilling, but some complex was detected in the periodontal tissue along the incisor up to 5 mm away from the bony window. Gold particles accumulated within clotted matrix (Figure 12A) and in endosomal/lysosomal elements of macrophages and neutrophils in the bony defect. Periodontal fibroblasts in the proximity of the window (Figures 12 and 13A) and, less frequently and intensely, pulp cells (Figure 13B) also showed the intracellular presence of tracer. No gold particles were detected in the enamel organ, in the contralateral tooth, or in other distant tissues sampled.

Discussion

Despite efforts to elucidate the regulatory role of matrix molecules and growth factors in tooth and bone formation, there are still many questions regarding the molecular mechanisms that underlie their formation. To address these issues, we have developed an in vivo

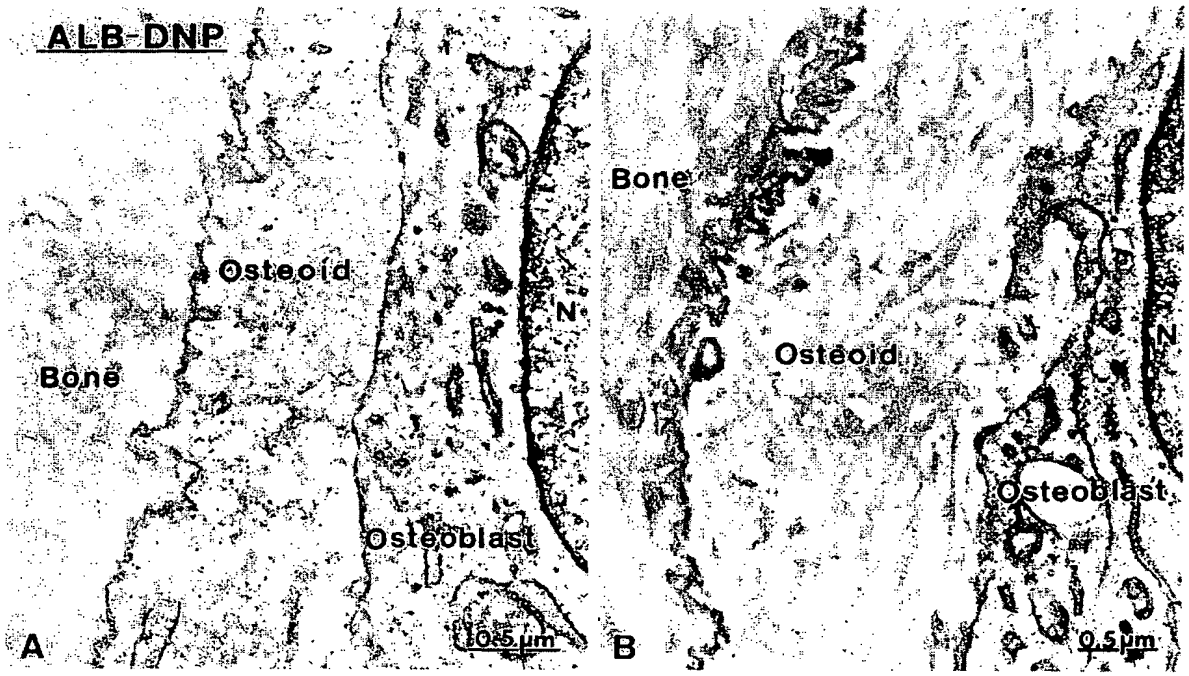

Figure 10 Albumin-dinitrophenol (ALB-DNP) is immunodetected in osteoid of the (A) treated hemimandible but not on the (B) contralateral side, indicating that there has been minimal recirculation of the tracer during the 24-hr infusion interval. N, nucleus.

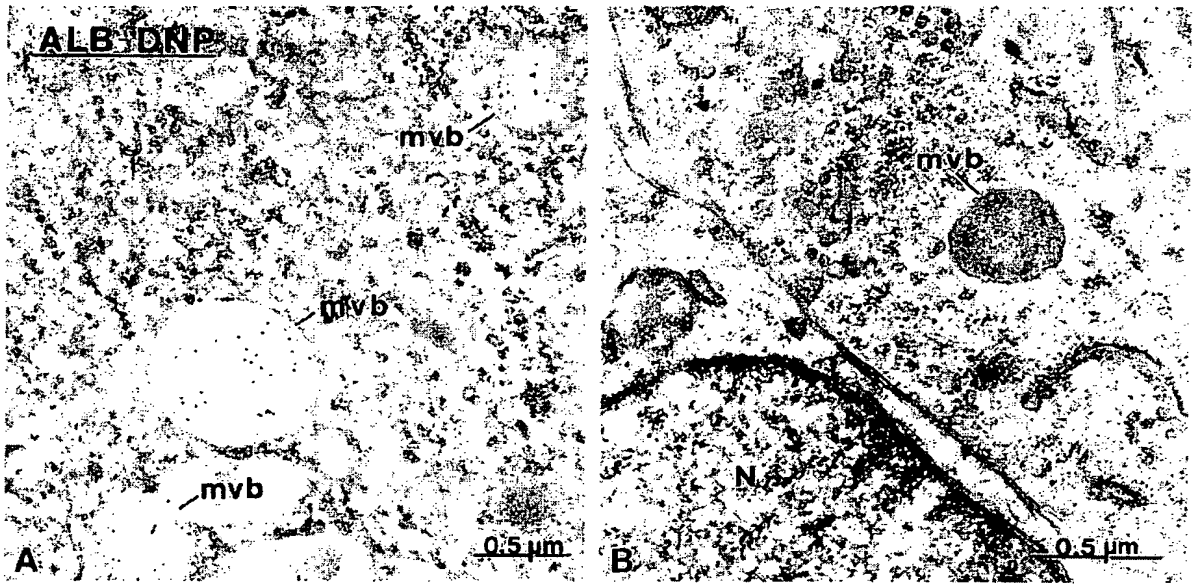

Figure 11 (A,B) Immunocytochemical preparations illustrating the presence of albumin-dinitrophenol (ALB–DNP) tracer in multivesicular bodies (mvb) of early secretory stage ameloblasts. Because these cells are separated from the pulp by mineralized layers of dentin and enamel, the endocytosed molecules must have diffused through the multiple cell layers of the enamel organ in order to reach the ameloblasts. (A) Nonosmicated; (B) osmicated. N, nucleus.

approach to experimentally access and manipulate the odontogenic organ of the rat incisor and its associated periodontal tissues. This tooth was chosen because it offers the possibility to investigate developmental processes and the deposition of both collagenous and noncollagenous mineralized matrices in a well-defined temporospatial sequence. An osmotic minipump, connected to a bony window in the alveolar bone overlying the apical end of the tooth, allowed controlled and continuous administration of experimental agents to the tooth organ and its surrounding tissues.

To avoid complications associated with previous experimental approaches for accessing the tooth organ (see Introduction), we positioned our surgical access on the buccal side of the alveolar bone, overlying the periodontal tissue just posterior to the apical end of the mandibular incisor. This site allows access to both the tooth organ and a zone of active bone remodeling along the posterior wall of the hemimandible. From this site, the experimental agents can diffuse to the enamel organ and its adjacent structures via the periodontal space separating the alveolar bone and the tooth. Damage to the odontogenic organ was observed in very few cases of operated animals and was, in general, due to anatomic variability. Most of the damage was confined to the apical end of the tooth and did not appear to significantly alter tooth formation and/or eruption. Damage to the enamel organ in some cases resulted in the production of a matrix resembling that found at the enamel-free area on the cusp tips of rodent molars, an observation relevant to the proposed epitheliomesenchymal transformation of enamel organ cells during odontogenesis (Bosshardt and Nanci 1998).

In contrast to systemic injection or local microinjection, with minipumps it is possible to deliver relatively large volumes of an experimental agent over a precise period of time. Vinblastine sulfate was infused over a 3-day period to obtain local tissue alterations without any systemic effects. The changes obtained in the enamel organ are consistent with previously published data using local or systemic injection of vinblastine (Moe and Mikkelsen 1977; Miake et al. 1982; McKee and Warshawsky 1984; Nanci and Warshawsky 1984; Nanci et al. 1987). In addition, alterations induced in the pulp, dentin, cementum, and periodontal tissue indicate that the drug diffuses locally and demonstrate the efficacy of our approach in targeting all the tissues of the tooth organ. Most importantly, the contralateral untreated tooth and distant tissues, such as duodenum, showed no sign of being affected by the drug, thereby confirming that restricted and localized effects can be obtained. Indeed, the treated incisor was ~1 mm shorter than the contralateral tooth, an amount

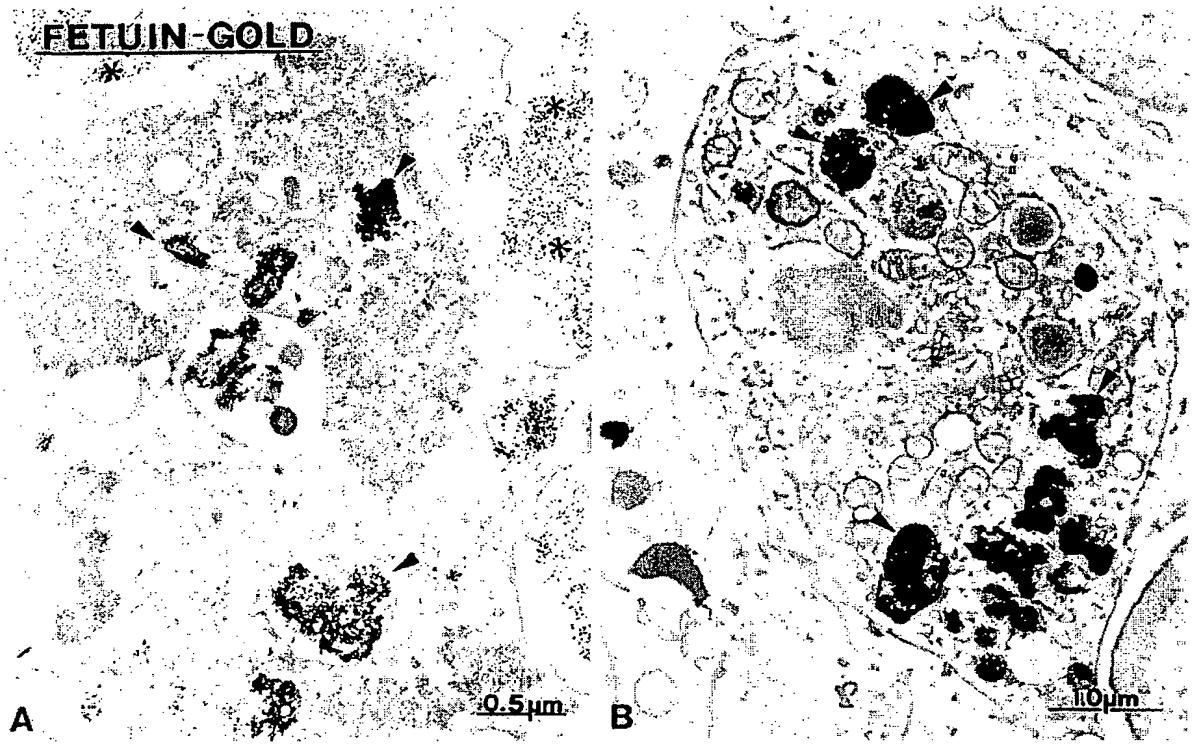

Figure 12 Fetuin–gold complex (A) binds to the extracellular matrix (asterisks) and is heavily taken up into endosomal/lysosomal elements (arrowheads) by inflammatory cells such as (A) neutrophils and (B) macrophages at the site of the bony window.

within the range but short of the expected eruption distance (1.9 mm) for mandibular incisors of rats weighing about 100 g during a 3-day interval (calculated from Smith and Warshawsky 1975b). This difference suggests that the treatment reduced but did not stop tooth eruption. Although some diffusion of infused products into the circulatory system is likely to occur, the fact that very low amounts are required to obtain a local effect greatly reduces the potential and intensity of any systemic side effects. Even in the case of DNP–albumin, a circulating serum protein, there was no significant systemic redistribution.

It is generally accepted that the presence of protein outside the cell stimulates the ingestion of solutes from the interstitial fluid (Alberts et al. 1994). The presence of tracers such as DNP–albumin and fetuin–gold in endosomal/lysosomal elements of ameloblasts, odontoblasts, osteoblasts and fibroblasts is consistent with the notion that most cells exhibit pinocytotic activity. Because secretory stage ameloblasts are separated from the pulp by mineralized layers of dentin and enamel, the presence of DNP–albumin in these cells demonstrates that relatively large proteins can diffuse into the multiple cell layers of the enamel organ, as previously shown after systemic injection (Nanci et al. 1996a). Accessibility of infused products to the various cells of the tooth and associated periodontal tissues is of utmost importance for gene activation/inactivation studies ("local" knockout or transgenic) using molecular probes. Fetuin–gold complex is a larger tracer molecule that was found mostly in the extracellular matrix and was intensely phagocytosed by inflammatory and fibroblastic cells in the immediate vicinity of the bony window. However, it did not diffuse across the enamel organ to be picked up by ameloblasts. Nevertheless, some fetuin–gold was detected in fibroblasts of the periodontal tissue as far away as 5 mm from the window. These tracer results indicate that the extent of tracer dissemination may vary depending on its size, its nature, its interaction with matrix proteins, and its ability to elicit an inflammatory response.

In conclusion, the data presented herein validate our experimental approach and show that the various cells of the tooth, as well as those of the surrounding tissues, can be targeted. It can be advantageously applied to manipulate in vivo, using various therapeutic

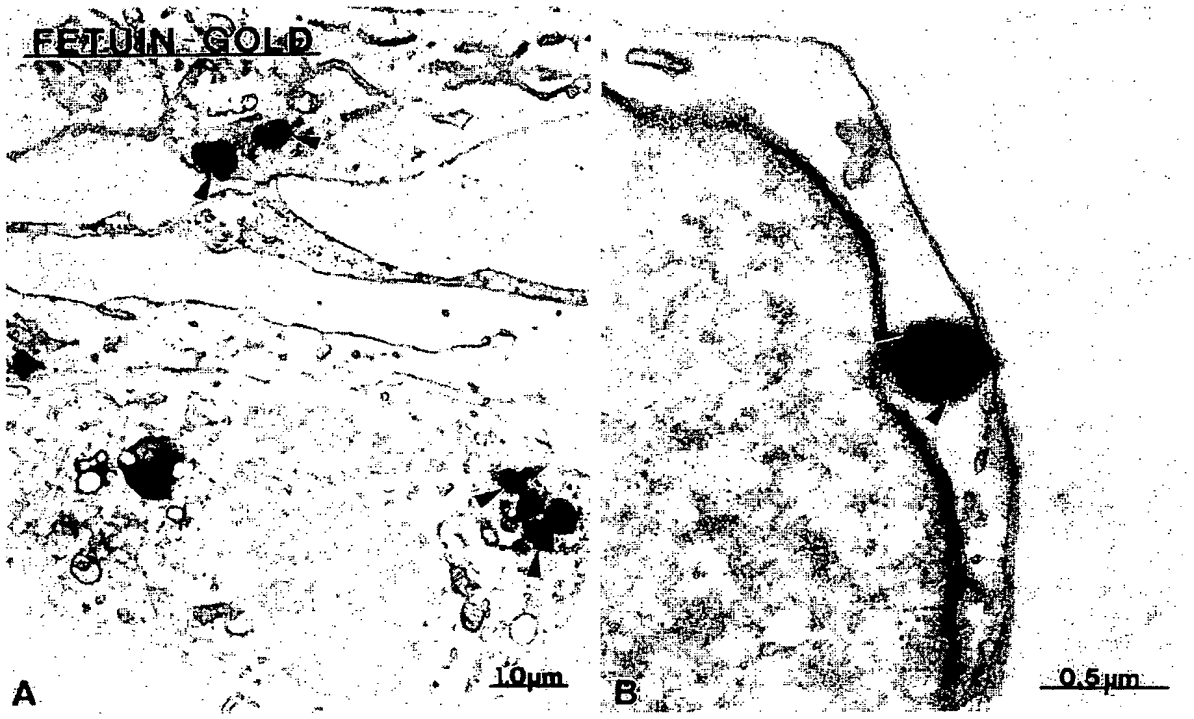

Figure 13 Despite their relatively large size, fetuin–gold complexes can be found (A) along the enamel organ and (B) in the pulp, where they are endocytosed and directed to endosomal/lysosomal elements (arrowheads) of connective tissue cells.

agents, the complex cellular and extracellular matrix events involved in the formation of both collagenous and noncollagenous calcified tissues. The ability to interfere with the genes responsible for the production of target proteins of the odontogenic organ and associated tissues may help advance our understanding of mineralized tissue formation and pathological alterations. Application of molecular probes through the bony window offers the possibility to activate/inactivate gene products locally and selectively for studies of function. Such an approach is potentially less time consuming and costly than the genetic engineering of knockout or transgenic animals, and may be particularly valuable in cases where genetic alterations result in a lethal phenotype. Enamel molecular probes have already been used in vitro (anti-sense; Slavkin 1995) and in vivo by gross local injection (ribozymes; Lyngstadaas et al. 1995) but these have not yet been extensively tested, in part due to their toxicity or generalized effects when injected systemically.

Acknowledgments

Supported by a grant from the Medical Research Council of Canada to AN. We are grateful to M. Fortin for excellent technical assistance, Dr L. Ghitescu for preparing and donating the dinitrophenylated albumin, and Nissei Sangyo Canada for use of the variable-pressure scanning electron microscope.

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